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13. ABSTRACT (Maximum 200 Words)

Accmululating evidence indicates that BRCA1 is a component of large molecular weight complexes. BARD1-BRCA1 is reported to be one such complex containing BRCA1. To date, there has been no attempt to purify BARD1-containing complexes. Biochemical purification can yield valuable insights into the polypeptide composition and the functional role of multiprotein complexes. Although the genetic approaches have been successful in defining the genes that are mutated in breast cancer, functional understanding of the protein product of these genes requires biochemical studies. Biochemical analysis of the gene products of BARD1, BRCA1 and BRCA2 will not only reveal their normal cellular function but also indicate the functional defects associated with the mutated proteins. Although such biochemical approaches have not been applied to studies of breast cancer, they have been successfully utilized in understanding complex cellular processes such as transcriptional regulation. We will isolate and functionally define the BARD-BRCA1-containing complex. We hypothesize that BARD1 plays a role in maintenance of genome stability through its interaction with the BRCA1. We will use biochemical techniques that have been instrumental in increasing our understanding of the transcriptional machinery, and that have not yet been fully utilized in studies of breast cancer, to isolate the BARD1-BRCA1 complex. We intend to identify BRCA1-associated proteins that are altered as the result of mutations in BRCA1 protein.

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Introduction:

We are using biochemical techniques to define the mutli-protein complexes that contain the breast cancer causing gene BRCA1 and BARD1. These studies are aimed at elucidating the precise biochemical mechanism of the BRCA1-BARD1 complex. This should lead to a better understanding of the underlying cause of breast cancer. Moreover, identification of novel BRCA1-BARD1 interacting components should lead to uncovering of genes whose mutations may cause breast and ovarian cancers.

BODY:

Task 1. Isolate and define the molecular characteristics of the BRCA1-BARD1 complex, using affinity purification by BARD1 antibodies. (months 1-18). This task has been accomplished and was reported on in the previous progress report.

Task 2. Analyze the polypeptide composition of BARD1-BRCA1 complex purified from cells carrying mutations in BRCA1 (months 18-30).

Cancer causing truncation of BRCA1 abrogates the association of BRCC36 and BRE.

We asked whether the truncation of BRCA1 protein, which may occur as a result of cancer causing frameshift mutations in exon 11, will result in the loss of BRCC36 or BRE association. To obtain a truncated BRCA1 complex, we constructed a 293-derived cell line expressing a truncated (1-509) BRCA1. As a control we also constructed cell lines expressing truncated form of BARD1 (1-398). Both truncated protein complexes were purified and the resulting polypeptides were analyzed for protein composition. While truncation of BARD1 did not affect the association of any of the components of

the complex, BRCA1 truncation completely abrogated the association of BRCC36, BRE and reduced the association of both BRCA2 and RAD51 with BRCC (Fig. 1A). In contrast, truncations of either BRCA1 or BARD1 did not affect the association of p53 and BRCC. These results correlate the loss of function of truncated BRCA1 protein with the loss of several key regulatory components of BRCC.

BRCC36 interacts with exon 11 of BRCA1

To ask whether BRCC36 can directly interact with BRCA1, six fragments of BRCA1 spanning the open reading frame were produced in bacteria and were tested for their association with recombinant BRCC36 also produced in bacteria (Fig. 1B). To address whether BRCC36 association with BRCA1 also extended to other JAMM domain containing proteins, the Jab1/CSN5 subunit of signalosome was also produced in recombinant form and was used in the protein-protein interaction assay. Consistent with the above results obtained from the truncating mutation of BRCA1, BRCC36 can specifically associate with fragments 3 and 4 located in exon 11 (Fig. 1C). This association is specific for BRCC36 since Jab1/CSN5 did not associate with any fragments of BRCA1 protein (Fig. 1C). Together these data indicate a direct interaction of BRCA1 and BRCC36 which is lost following cancer-causing truncations of BRCA1.

Task 3. Molecular cloning of BRCA1-associated polypeptides (months 24-36).

BRCC36 and BRCC45 are novel subunits.

Mass spectrometric sequence analysis of the 36 kDa band (BRCC36) identified this protein as the polypeptide encoded by the *c6.1A* gene. The BRCC36/*c6.1A* gene is located at the Xq28 locus, a chromosomal break point in patients with pro-lymphocytic

T-cell leukaemia. BRCC36 displayed sequence homology with human Poh1/Pad1 subunit of the 26S proteasome, and with the subunit 5 (Jab1) of the COP9 signalosome (Fig. 2A). This homology is in the recently described Jab1/MPN or the JAMM domain. Despite its homology to POH1 and Jab1, BRCC36 represents a distinct branch in the evolutionary tree. Interestingly, the COP9 complex has been shown to regulate the activity of the SCF ubiquitin ligase complex. Specifically, the Jab1 subunit of the COP9 complex is reported to modulate the degradation of p27 protein. BRCC45 protein correspond to the brain- and reproductive-organs-specific gene, BRE. BRCC45/BRE does not display homology to any other protein in the human genome.

To rigorously establish the association of BRCC36, BRE and BRCA1/BARD1, we developed a 293 derived cell line stably expressing Flag-BRCC36. Isolation of Flag-BRCC36 by Flag-affinity purification demonstrated the stable association of BRCC36 with the other components of BRCC (Fig. 2B). It is noteworthy that we detected the endogenous BRCC36 in the Flag-BRCC36 affinity eluate, indicating the presence of more than one BRCC36 protein per BRCC.

Task 4. Analyze the functional activity of BARD1-BRCA1 complex purified from cells carrying mutations in BRCA1 (18-36). This task is being pursued as delineated in the original application.

Figure Legends.

Figure 1. Cancer causing truncations of BRCA1 abolished the association of BRCC36 and BRCC.

(A) Western blot analysis of complexes purified from full-legth Flag-BARD1, truncated Flag-BRAD1 (1-398) and truncated Flag-BRCA1 (1-509) stable cell lines using antibodies to the right of the panel. (B) Diagramatic depiction of the six GST fragments of BRCA1 used for the protein-protein intercation analysis shown in C. (C) GST pull down experiments demonstrating the association of BRCC36 and not the Jab1 subunit of signalosome with fragments 3 and 4 of BRCA1 shown in panel B.

Figure 2. BRCC36 and BRCC45 are novel components of BRCC. (A) Diagramatic alignment of human BRCC36 (P46736), human Poh1 (AAC51866) and human Jab1 (NP_006828). The numbers represent amino acid sequences and the shaded box reflect the homologous domain. The numbers above the shaded boxes represent percent identity and similarity of each protein to human BRCC36. (B) Western blot analysis of the Flag-BRCC36 affinity eluate. Antibodies used are shwn to the right of the panel.

Key Research Accomplishments:

1-Isolated the BRCA1-BARD1 complex from human cells.

2-Determine the polypeptide composition by MS/MS sequencing.

3-Characterized the ubiquitin ligase activity of BRCA1-BARD1 complex.

4-Cloning of BRCC36 and BRCC45.

5-Estabilishing stable lines expressing BRCC36 and BRCC45.

6-Analysis of polypeptide composition of BRCA1 complexes containing the truncated forms of BRCA1 or BARD1.

Reportable Outcomes:

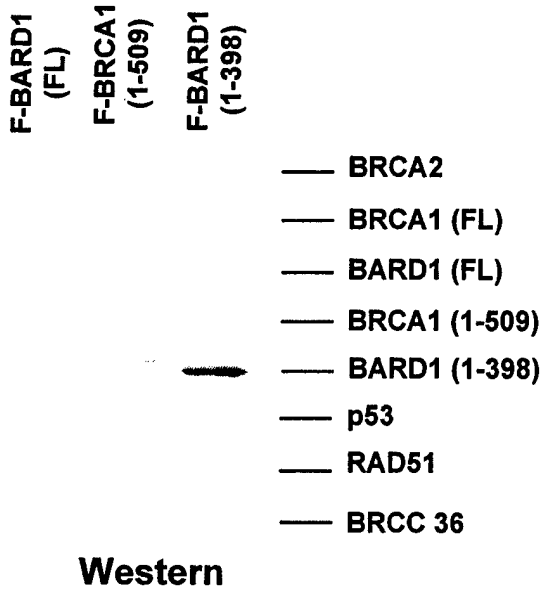
Isolation of novel genes whose mutations are involved in breast cancer development is certainly an out come of these studies. Currently we are performing a number of experiments aimed at determining whether BRCC36 and BRCC45 are mutated or aberrantly expressed in breast cancer. Also we are submitting patent applications on the genes that were identified by microsequencing analysis.

Conclusions: BRCC represents the first BRCA1/BRCA2/BARD1-containing complex that displays an E3 ubiquitin ligase activity. Although BRCC contains BRCA1, BRCA2 and BARD1, there are a number of differences in the polypeptide composition of BRCC and complexes reported by others. Importantly, with the exception of sub-stoichiometric amounts of RAD51, BRCC does not stably associate with any known DNA repair factors. Indeed, fractionation of BRCC by ion exchange chromatography results in the separation of the bulk of RAD51 from the other components of the complex (data not shown), suggesting a weaker interaction of RAD51 with the core-subunits of BRCC. We therefore favor the contention that BRCC may not be playing a direct role in DNA repair, but through its E3 ubiquitin ligase activity BRCC can regulate factors involved in DNA repair.

We show that BRCC36 directly interacts with a fragment encoded by exon 11 of the BRCA1 gene. Isolation of BRCC from cell lines expressing BRCA1 with a truncation in this region (a C-terminal truncation), which mimics the cancer-causing

mutations of BRCA1, resulted in the loss of BRCC36 from the BRCC. Interestingly, C-terminal truncations of BARD1 did not have a destabilizing activity on the components of BRCC. This may indicate that, while cells may be able to tolerate the truncating mutations of BARD1, similar mutations in BRCA1 leads to a disruption of BRCC integrity and a concomitant deregulation of growth stimulatory pathways.

A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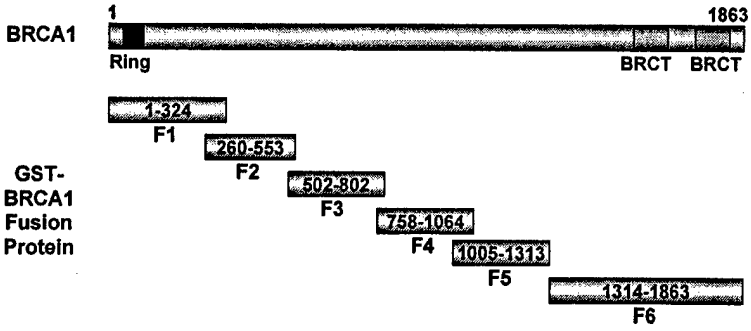
F-BRCA1 (1-509)
F-BARD1 (1-398)
BRCA2
BRCA1 (FL)
BARD1 (FL)
BRCA1 (1-509)
BARD1 (1-398)
p53
RAD51
BRCC 36
Western

B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1863
Ring
BRCT
BRCT
1-324
F1
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F2
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F3
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1005-1313
F5
1314-1863
F6
GST-BRCA1 Fusion Protein

C

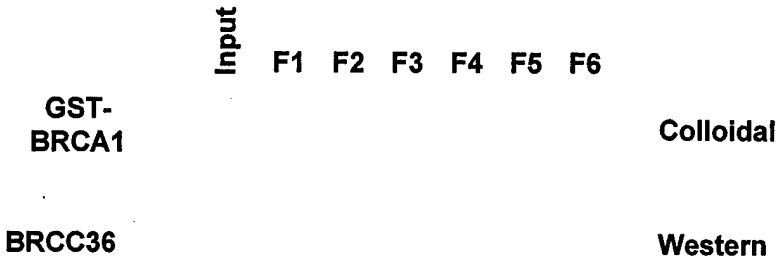
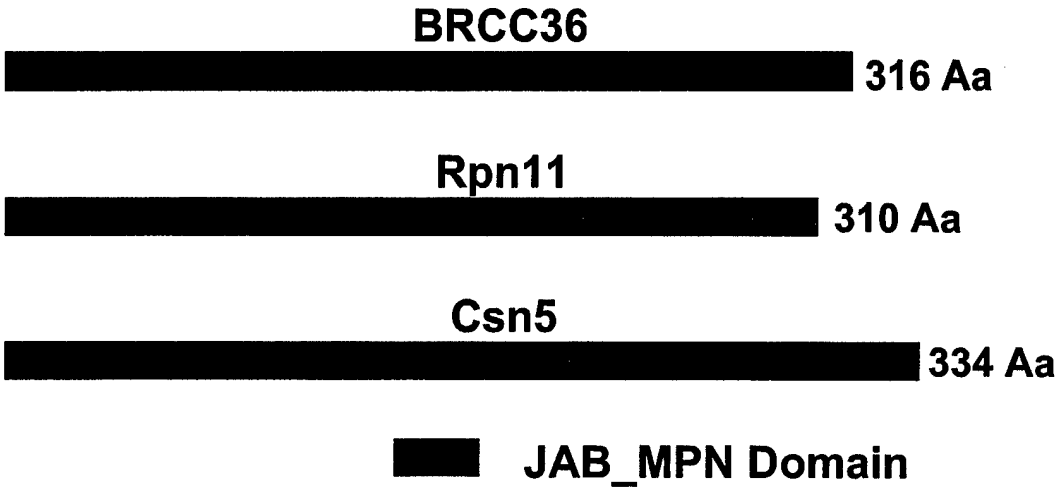

Input
F1
F2
F3
F4
F5
F6
GST-BRCA1
Colloidal
BRCC36
Western

Figure 1

A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Csn5
334 Aa
JAB_MPN Domain

B

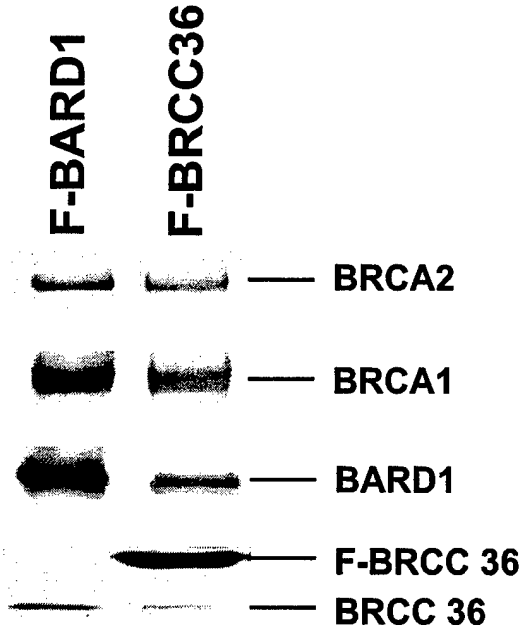
F-BARD1
F-BRCC36
BRCA2
BRCA1
BARD1
F-BRCC 36
BRCC 36

Figure 2